IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

ATTY.'S DOCKET: WALLACH1D

In re Application of:	)	Art Unit: 1646
	)	
David WALLACH, et al.	)	Examiner: J. Dong
	)	
Appln. No.: 10/036,434	)	Washington, D.C.
	)	
Date Filed: January 7, 2002	)	Confirmation No.: 4966
	)	
For: TUMOR NECROSIS FACTOR INHIBITORY PROTEIN …	)	

DECLARATION UNDER 37 CFR §1.132 OF RIK DERYNCK

Honorable Commissioner for Patents
U.S. Patent and Trademark Office
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Sir:

I, the undersigned Rik Derynck, Ph.D., hereby declare and state as follows.

I have been responsible for running a research lab since 1981, and during these 25 years have combined molecular biology, cell biology and protein biochemistry in my approaches to scientific questions. The integration of these different disciplines and areas of research is an essential aspect of running a successful laboratory. Indeed, these disciplines are becoming increasingly integrated, and, for example, protein biochemical approaches are now often fully

combined with molecular biology approaches to address molecular biology or cell biology problems.

Throughout these 25 years of running my lab, I have stayed abreast of the progress in protein biochemical approaches, to the extent that this is needed for the integration of these approaches into my lab's research. Essential aspects of my responsibilities include the need to be informed of what can and cannot be done through purification of proteins that are of interest to my research, and the need to make judgments and to provide guidance to my students, postdoctoral fellows and technicians on how to properly approach protein identification and characterization projects.

Accordingly, I have hands-on experience with protein purification and have provided guidance to others working in my lab on protein purification projects. Starting with my undergraduate training, I received solid training in biochemistry and analytical biochemistry. In addition, I started my Ph.D. thesis research with the purification of the enzymes HindII and HindIII, and EcoRII. My subsequent experience with protein purification, as documented in the literature, can be illustrated with the following examples of papers from my lab.

Derynck, R., Roberts, A.B., Winkler, M.E., Chen, E.Y., and Goeddel, D.V. (1984) "Human transforming growth factor-α: precursor structure and expression in *E. coli*." *Cell* 38, 287-297.

Brachmann, R., Lindquist, P.B., Nagashima, M., Kohr, W., Lipari, T., Napier, M., and Derynck, R. (1989) "Transmembrane TGF-α precursors activate EGF/TGF-α receptors." *Cell* 56, 691-700.

Graycar, J. L., Miller, D.A., Arrick, B.A., Lyons, R.M., Moses, H.L., and Derynck, R. (1989) "Human transforming growth factor β3: recombinant expression, purification and biological activities in comparison with transforming growth factors -β1 and -β2." *Molec. Endocrin.* 3, 1977-1986.

Balentien, E., Han, J. H., Thomas, H. G., Wen, D., Samantha, A. K., Zachariae, C. O., Griffin, P., Brachmann, R., Wong, W. L., Matsushima, K., Richmond, A., and Derynck, R. (1990) "Recombinant expression, biochemical characterization and biological activities of the human MGSA/gro protein." *Biochemistry* 29, 10225-10233.

Thomas, H.G., Han, J.H., Balentien, E., Derynck, R., Bordoni, R., and Richmond, A. (1991) "Purification and characterization of recombinant melanoma growth stimulating activity." *Meth. Enzymol.* 198 C, 373-383.

Kuo, A., Zhong, C., Lane, W.S. and Derynck, R. (2000) "Transmembrane transforming growth factor-α tethers to the PDZ-domain containing, Golgi-membrane-associated protein p59/GRASP55." *EMBO J.* 19, 6427-6439.

My Curriculum Vitae is submitted herewith as Exhibit A.

I was informed that, in the prosecution of the above-identified application, the examiner alleges that "there is no evidence indicating what had been accomplished in the instant application, i.e. partial N-terminal sequencing of the polypeptide, could not have been achieved by using Seckinger's purified inhibitor of TNF-α." I understand that the "Seckinger" referred to by the examiner is Seckinger et al., *J. Exp. Med.*, 157:1511-1516 (1988) (hereinafter, Seckinger et al. (1988)). It is my opinion that substantial evidence exists that the impure inhibitor of TNF-α obtained by Seckinger et al. (1988) could not have been used to obtain partial N-terminal sequencing of the polypeptide for all of the reasons set forth below.

Seckinger et al. (1988) reported fractionation of a urinary protein mixture using Sephacryl S-200, and a fractionation by Mono P chromatofocusing. These fractionations are presented in the publication as separate and alternative fractionation approaches, and not as sequential steps toward the purification of the TNF inhibitor. Seckinger et al. (1988) neither provided evidence demonstrating generation of substantially purified TNF inhibitor nor did they claim to be in possession of a substantially purified TNF inhibitor. In fact, no sequence information for the TNF inhibitor was reported in his publication.

On the contrary, Seckinger et al. (1988) described experiments resulting in an inhibitory activity eluted from the gel (Sephacryl S-200) in a single peak, and possessing maximal

inhibitory activity showing an apparent molecular mass of 40-60 kD (p. 1512 and Fig. 1). In a different experiment, salt precipitated urine (not the gel filtrated urine) was chromatofocused and the bulk of the TNF inhibitory activity (but not all) was found in fractions that eluted between pH 5.5 and 6.1.

In addition, Seckinger et al. (1988) clearly states (p 1515) in the discussion:

> We have found … a TNF-α inhibitory activity, whose nature remains to be determined by purification to homogeneity, many bands still being identified in SDS-PAGE of the Sephacryl-200 inhibitory fractions. Purification will enable us to establish a relationship with one or more putative inhibitors. [Emphasis added]

Thus, it is apparent from Seckinger et al. (1988) that these investigators were not in possession of a purified protein that could have been even partially sequenced.

The process of purification of a protein found in biological fluid is a challenging and complex matter. The human genome is thought to encode between 30 and 35 thousand genes. While each gene encodes at least one polypeptide, alternative transcription and translation initiation, as well as differential splicing, often result in a heterogeneity of proteins that, while related, nevertheless have different identities, different sizes and charges, and thus overall different biochemical properties. Additional heterogeneity is often created by post-translational modifications, such as

phosphorylation, O-glycosylation, ubiquitylation or sumoylation for intracellular or cell-associated proteins, or different types and degrees of N-glycosylation of extracellular and secreted proteins, all of which confer different molecular weights and charges, and form the basis for extensive complexities in protein biochemical properties. Taken together, the number of different proteins with different biochemical properties exceeds by far the number of genes. Since the biochemical properties are the basis for protein purification, one can easily imagine the enormous task one faces in purifying a protein from a complex mixture of proteins, as encountered in any biological fluid, such as saliva and urine. Indeed, thousands of different proteins are found in these fluids, all of which have different properties and identities, and all of which are present in different amounts.

In order to derive sequence information directly from one such protein, the protein of interest needs to be purified to an extent that it is the predominant protein in the preparation that will be subjected to the sequencing method. This is not a trivial undertaking since the starting material for purification usually contains thousands of proteins and the protein of interest is thus only one of the thousands with an abundance that is usually only a small fraction of a percent of the total protein content, albeit unknown at the time of purification. Thus, any protein purification protocol designed to yield a protein preparation in which the protein of interest

is the predominant one, requires multiple, sequential fractionation and purification steps. A single protein purification step is insufficient to provide a protein of sufficient purity for sequencing. In fact, two sequential purification steps would not be expected to suffice either. Indeed, the combination of multiple sequential steps, each based on different fractionation or purification parameters, is often required for purification to a sufficient degree for sequencing.

With the advent of extensive databases of protein sequences in the late 1990's and the sequence information that has been generated in the last decade through the multiple approaches to identify all human genes in the human genome, today it is possible to obtain sequences from mixtures of proteins in which the protein of interest does not have to be the predominant protein, yet still has to be a major component. Thus, the complexity of the protein mixture has to be such that it contains only a few proteins, each of which has to be sufficiently abundant in relative and absolute terms that it can generate peptide sequences. Thus, such mixtures need to have undergone extensive purification, away from other proteins, and extensive enrichment of the protein of interest. However, this capability to obtain sequence information from low complexity mixtures of proteins simply did not exist in the early 90's or before when scientists were attempting to isolate an inhibitor of TNF-α.

As indicated above, sequential fractionation and purification steps are necessary in order to obtain a substantially purified protein of interest at a purity and in quantities sufficient for successful peptide sequencing. However, even sequential fractionation and purification steps are not necessarily sufficient. All of this can best be illustrated reviewing the results of three different research groups reporting the purification of the TNF inhibitor in the same timeframe as the present applicants. On information and belief, all of the publications referred to below, except as otherwise indicated below, are already of record and available to the examiner in this case.

(1) In order to achieve substantially pure protein that is capable of being sequenced, Engelmann et al., *J. Biol. Chem.*, 264:11974-11980 (1989) (hereinafter Engelmann, et al. (1989)) used, sequentially, (a) concentration through filtration, (b) cation exchange chromatography on CM Sepharose, (c) cation exchange HPLC on Mono S, (d) anion exchange HPLC on Mono Q, finally followed by (e) reversed phase HPLC on Aquapore RP300. This purification protocol yielded substantially purified protein from which Engelmann et al. were able to determine sequence information for the TNF inhibitor. This is the procedure described in the above-identified application.

(2) In contrast, Seckinger et al., *J. Biol. Chem.*, 264:11966-11973 (1989) (hereinafter, Seckinger et al. (1989)) used, sequentially, (a) concentration and fractionation using filtration and ammonium sulfate precipitation, (b) anion

exchange chromatography using DEAE Sephadex, (c) cation exchange chromatography using sulphopropyl Sephadex, (d) gel filtration using Sephacryl S200, (e) TNF-α affinity chromatography, finally followed by (f) Pro-RPC reversed phase FPLC. Although this purification protocol yielded substantially purified protein, it nevertheless failed to yield sequence information for the TNF inhibitor.

(3) In further contrast, Olsson et al., *Eur. J. Haematol.*, 42:270-275 (1989) (hereinafter, Olsson, et al. (1989)) used, sequentially, (a) concentration through filtration, (b) anion exchange chromatography using DEAE Sephacel, (c) TNF-α affinity chromatography, finally followed by (d) Pro-RPC FPLC. The authors report that this yielded a 1.1 million-fold purification. The resulting protein preparation was a substantially purified protein and was sufficiently purified to generate sequence information for the TNF inhibitor.

(4) During the next year, Seckinger et al., *Eur. J. Immunol.*, 20:1167-1174 (1990) (hereinafter, Seckinger, et al. (1990) - a copy of which is submitted herewith as Exhibit B) reported that they used, sequentially, (a) concentration by ammonium sulfate fractionation, (b) TNF-α affinity chromatography, (c) cation exchange FPLC on Mono S, (d) Pro-RPC reversed phase FPLC, finally followed by (e) microbore reversed phase HPLC. This purification method yielded substantially purified protein from which these investigators were able to determine sequence information for the TNF inhibitor.

These reports of substantially purified protein preparations for the TNF inhibitor, only three of which yielded partial N-terminal sequencing, stand in contrast with two other earlier reports from two of the same groups.

(5) Seckinger et al. (1988), the reference cited by the examiner and already mentioned above, reported a fractionation of the urinary protein mixture using Sephacryl S200, and a fractionation by Mono P chromatofocusing. These fractionations were carried out as separate and alternative approaches, and not as sequential steps toward the purification of the TNF inhibitor. As stated above, Seckinger et al. (1988) provided no evidence demonstrating possession of a substantially purified TNF inhibitor. In fact, they did not report sequence information for the TNF inhibitor. Please note that these investigators are the same group who generated substantially purified TNF inhibitor using a sequential purification approach but who were unable to provide sequence information even in 1989 (Seckinger et al. (1989). It wasn't until Seckinger's 1990 publication (Seckinger et al. (1990)) that his team of investigators was able to provide such sequence information.

(6) Finally, Peetre et al., *Eur. J. Haematol.*, 41:414-419 (1988) (hereinafter, Peetre et al. (1988)) used, sequentially, (a) concentration through filtration, (b) anion exchange chromatography using DEAE Sephacel, finally followed by (c) Sephadex G-75 chromatography. They reported only a 62-fold partial purification, and no sequence information for the

TNF inhibitor. Please note that these investigators are the same group as in Olsson et al. (1989), who, about one year later, reported sequence information, based upon a different sequential purification protocol that resulted in a 1.1 million-fold purification of the TNF inhibitor protein.

It is clearly evident from examination of the above references that the product of the Seckinger et al. (1988) publication was insufficiently pure to allow sequencing using the technology available circa 1990.

It should also be clear from the above recitation that different purification methods can and do result in completely different degrees of purification.

Any purification step based on charge or on size of the protein essentially represents a fractionation, whereby many proteins with similar charges or sizes, respectively, are co-fractionated. While each fractionation method represents an important step in its own right, it is only the sequential combination of multiple fractionation steps that will yield a protein of sufficient purity to allow sequencing. For example, the DEAE-Sephacel step of Olsson et al. (1989) represents a 4-fold purification, whereas the CM Sepharose step of Engelmann et al. (1989) yielded a 20-fold enrichment.

FPLC and HPLC methods, based on fractionation by size or charge, represent powerful purification steps, but again they are insufficient to provide purification in a single step and need to be combined in sequence with other purification steps to yield a protein sufficiently pure for sequencing. For

example, the Pro-RPC FPLC step provides a close to 40-fold purification for Olsson et al. (1989), and a 27-fold purification for Seckinger et al. (1990), whereas the Mono S column provides 1.8-fold purification for Engelmann et al. (1989) and a 34-fold purification for Seckinger et al. (1990).

More powerful purification can be achieved using affinity chromatography, whereby in this case the inhibitor can be purified through its exquisite selectivity and affinity for TNF. Thus, TNF affinity chromatography provided a 114-fold purification in the protocol of Seckinger (1990) and a remarkable 9000-fold purification in the protocol of Olsson (1989). It should be noted, however, that even such a dramatic purification step as TNF-α affinity chromatography is insufficient to yield a protein purification of sufficient purity that it becomes suitable for sequencing. Thus, even affinity-based purification needs to be combined with additional purification or fractionation steps.

The activity of a protein represents a very powerful parameter for the identification and purification of a protein. In this case, the ability of the protein of interest to neutralize the activity of TNF, the key identifier of activity of this protein, allows for the sensitive detection and purification of the protein. However, the activity of the protein *per se* does not provide *a priori* a parameter to estimate its purity. Thus, a single peak of protein activity using one or another purification step does not provide any indication of and is not a reflection of the extent of its

purity. To illustrate this for the TNF inhibitor, Engelmann et al. (1989) report that the protein used for sequencing had a 54,000-fold purification, whereas the fold purification for Seckinger et al. (1990) was 81,000, and for Olsson et al. (1989) was 1,100,000, before it was used for sequencing.

Evaluation of all these data clearly shows that the protein could already be clearly detected as a single, well-resolved peak of activity, thus devoid of other proteins that might interfere with activity assays or score similarly in such assays, when the protein of interest still had to undergo substantial further purification and thus well before it could be used for sequencing. Thus, Peetre et al. (1988) detected the TNF inhibitor as a single peak of activity following a 4.9-fold purification, whereas they (Olsson et al. 1989) needed a 1.1 million-fold purification to obtain sequence for the TNF inhibitor. Similarly, Seckinger et al. (1990) demonstrate a single, well-resolved peak of TNF inhibitor activity at a purification step that resulted in 3,970-fold purification, even though 81,170-fold purification was needed to obtain purified protein for sequencing. Finally, Engelmann et al. (1989) demonstrate a clean, well-resolved peak of inhibitor activity in a purification step that yielded 88-fold purification, even though a 54,000-fold purification was needed to generate purified protein for sequencing. These results, together with the scores of these peaks in the activity assays, demonstrate that the inhibitor is clearly and unambiguously detected in preparations in which it represents less than

1/1000th of the total protein preparation. Thus, at such stage of purification, less than 1 per 1000 molecules was the inhibitor of interest, even though the protein presented itself as a well-resolved, "clean" peak of activity, devoid of any other proteins that interfered with its biological purity in this assay. Nevertheless, at such stage the protein is still very impure and substantial purification is then still needed before one has a protein that is biochemically sufficiently pure to allow its characterization by sequencing.

Reviewing the documents of interest in this case, Engelmann et al. (1989), Olsson et al. (1989) and Seckinger et al. (1990), three different groups, all report convincingly the purification of the TNF inhibitor that allows them to generate sequence information that identifies the inhibitor.

In contrast, the Peetre et al. (1988) paper, which is from the same group as the Olsson et al. (1989) paper, also provides a purification protocol, but no evidence is provided showing that the protein was purified or that it was sufficiently pure to allow peptide sequencing, nor were any such claims made. Indeed, the Olsson et al. (1989) paper, that provides identification of the protein by sequencing, reports a substantially different purification approach, essentially illustrating that the Peetre et al. (1988) purification protocol was not adequate in providing protein that could be successfully sequenced.

Similarly, the Seckinger et al. (1989) paper, published in the same issue of *J. Biol. Chem.* as the Engelmann

et al (1989) paper, also reports a purification protocol, but no evidence is provided that it was sufficiently pure to allow peptide sequencing. In fact, the Seckinger et al. (1990) paper, that provides identification of the protein by sequencing, reports a substantially different purification approach, essentially illustrating that the Seckinger et al. (1989) purification protocol was not adequate in providing protein that could be successfully sequenced.

In contrast to Seckinger et al. (1989), which pursued the purification of the TNF inhibitor, although it did not provide a protocol to generate sufficiently purified inhibitor for successful sequencing, Seckinger et al. (1988) did not provide any evidence for purification, nor was the generation of purified protein claimed by the authors. Instead, this paper reports two purification experiments, i.e., a fractionation of the urinary protein mixture using Sephacryl S200, and a fractionation by Mono P chromatofocusing. Each of these experiments presents a single fractionation step; no disclosure of the sequential use of these two methods was presented.

While the degree of purification or enrichment corresponding to these single steps is not reported, I want to make two points to illustrate the very low degree of purification:

(1) As summarized above, any single fractionation step that is not based on TNF affinity chromatography, as reported by Engelmann et al. (1989), Seckinger et al. (1990) or

Olsson et al. (1989), provides at best a 40-fold purification. If this would have been achieved by Seckinger et al. (1988), then the protein would still have to be more than 1000-fold further purified before it would be useful for protein sequencing. Indeed, Engelmann et al. (1989) and Seckinger et al. (1990) report the need for 54,000-81,000 fold purification in order to have protein that can be successfully sequenced.

(2) An evaluation of the activity profiles against the protein quantification profiles in both experiments illustrate that (a) the TNF inhibitor activity presents itself as a broad peak reflecting lack of well-defined or efficient fractionation, (b) these broad peaks coincide with a substantial fraction of the protein, corresponding to at least 20% of the total protein eluted from these columns.

In other words, the admittedly impure active fractions of Seckinger et al. (1988) correspond to at least 20% of the protein, suggesting a 5-fold enrichment. Since, again, a 54,000-81,000 fold purification is required to have protein of sufficient purity for sequencing, the data suggest that only 1 out of 10,000 molecules in the semi-purified fraction corresponds to the inhibitor. Even with the best technology of today, extensive purification of these biologically active protein mixtures would be required before one would even start considering sequence analyses.

Thus, I can opine with a very high degree of certainty, based on the above-described evidence, that the product of the Seckinger et al. (1988) publication was

insufficiently pure to allow sequencing using the technology available circa 1990.

The undersigned declares further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

2/21/2007	/Rik Derynck/
Date	Rik Derynck, Ph.D.

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